Normal and Abnormal Pathfinding of Facial Nerve Fibers in the Chick Embryo

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SUMMARY

Development of the facial nerve was studied in normal chicken embryos and after surgical disruption of ingrowing sensory facial nerve fibers at 38–72 h of incubation. Disruption of facial nerve fibers by otocyst removal often induced a rostral deviation of the facial nerve and ganglion to the level of the trigeminal ganglion. Cell bodies of the geniculate ganglion trailed their deviating neurites and occupied an abnormal rostral position adjacent to the trigeminal ganglion. Deviating facial nerve fibers were labeled with the carbocyanine fluorescent tracer DiI in fixed tissue. Labeled fibers penetrated the cranium adjacent to the trigeminal ganglion, but they did not follow the trigeminal nerve fibers into the brain stem. Rather, after entering the cranium, they projected caudally to their usual site of entrance and proceeded towards their normal targets. This rostral deviation of the facial nerve was observed only after surgery at 48–72 h of incubation, but not in cases with early otocyst removal (38–48 h). A rostral deviation of the facial nerve was seen in cases with partial otocyst removal when the vestibular nerve was absent. The facial nerve followed its normal course when the vestibular nerve persisted. We conclude that disruption of the developing facial pathway altered the routes of navigating axons, but did not prevent pathfinding and innervation of the normal targets. Pathfinding abilities may not be restricted to pioneering axons of the facial nerve; later-developing facial nerve fibers also appeared to have positional information. Our findings are consistent with the hypothesis that navigating axons may respond to multiple guidance cues during development. These cues appear to differ as a function of position of the navigating axon.

Keywords: **pathfinding, axon guidance, facial ganglion, cranial nerves, chicken (bird), development.**

INTRODUCTION

Axons grow along specific pathways to their appropriate targets. When outgrowing axons are manipulated to originate from ectopic sites they form aberrant pathways (Constantine-Paton, 1983), but many axons still reach their appropriate targets (Hamburger, 1961; Ghysen, 1978; Anderson, 1981; Lance-Jones and Landmesser, 1981; Moody and Heaton, 1983a; Pike, Melancon, and Eisen, 1992). These experiments suggest that navigating motor and sensory axons seek specific targets, presumably by responding to local cues (Lance-Jones and Landmesser, 1981). Specificity of pathfinding is determined quite early (Székely, 1959; Eide, Jansen, and Ribchester, 1982; Frank and Westerfield, 1982; Scott, 1986; Eisen, 1991). Thus, one would expect that the axons of placode-derived neurons may respond to different cues than axons of neural crest-derived cells.

Much of the recent work on pathfinding in the nervous system of vertebrates has focused on peripheral pathfinding of motor neurons and neural crest-derived sensory ganglia in the chick embryo (Lance-Jones and Landmesser, 1980, 1981; Landmesser, 1980; Lewis, Chevallier, Kieny, and Wolpert, 1981; Moody and Heaton, 1983c; Whitelaw and Hollyday, 1983a,b; Honig, Lance-Jones, and Landmesser, 1986; Landmesser and Honig, 1986; Scott, 1986). Studies on the pathfinding of neural crest-derived dorsal root ganglia have concluded that the fibers of these sensory neurons follow the

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pioneering axons of motor neurons to peripheral targets.

Sensory neurons of cranial ganglia originate from two distinct sources, neural crest and ectodermal placodes (Le Douarin, 1986). Little is known about pathfinding properties of placode-derived cranial ganglia. After the removal or transplantation of neural crest, fibers of placodal sensory neurons often fasciculate with adjacent cranial nerves and follow aberrant paths (Yntema, 1944; Hammond and Yntema, 1958; Hamburger, 1961; Noden, 1978; Moody and Heaton, 1983a,c). Placode-derived ganglia differ from neural crest-derived ganglia in many aspects including position, morphology, time of birth, time of neurite outgrowth, and responses to growth factors (Hamburger, 1961; Noden, 1978; D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983; Davies and Lindsay, 1985). Can placodal cells establish appropriate central projections when they are deprived of neural crest? Does their ability to form specific central projections depend on the time and site of entrance of nerve fibers into the medulla? To answer these questions, we investigated pathfinding abilities of the geniculate, placode-derived, facial ganglion in chick embryos.

Sensory cells of the facial nerve originate from two different sources. The geniculate (distal facial) ganglion derives from the ectodermal placode, whereas the proximal facial ganglion derives from the neural crest (D'Amico-Martel and Noden, 1983). Subsequently, the proximal facial ganglion fuses with the ganglion of the eighth nerve, and the facial and the vestibulocochlear nerves use a common pathway to enter the brain. The facial and vestibulocochlear nerves diverge when they enter the medulla to innervate distinct nuclei in the central nervous system (CNS). Otocyst removal eliminates the eighth nerve and ganglion and disturbs the normal pattern of cells in the proximal facial ganglion (Yntema, 1944), but it does not interfere with the generation of facial geniculate ganglion cells which are exclusively derived from the ectodermal placode (D'Amico-Martel and Noden, 1983). Thus, it is possible to examine pathfinding abilities of facial ganglion cells that are deprived of proximal structures which their processes normally encounter.

We studied the development of the facial nerve in the chick embryo to determine pathfinding abilities of these nerve fibers en route to their central targets after disruption of the facial–vestibulocochlear pathway. We show that after removal of the otocyst at 48–72 h of incubation (the approximate time of fusion of the facial–vestibulocochlear pathway) facial nerve fibers still proceed towards their normal targets, but often via a rostral deviation of the facial nerve and a rostral displacement of the geniculate facial ganglion cells to the level of the trigeminal ganglion. A preliminary account of our study has been presented in abstract form (Yang, von Bartheld, and Rubel, 1989).

MATERIALS AND METHODS

Fertilized eggs from White Leghorn chickens were obtained from a local supplier and incubated in 50%–60% relative humidity at 37.5°C. The normal development of the facial nerve was studied in 38 embryos [embryonic (E) day 2.5 to E12] with acetylcholinesterase histochemistry or by injecting neuronal tracers (DiI, DiO) into the peripheral facial nerve and its targets in fixed tissue. The development of the facial nerve was investigated in 20 embryos after unilateral otocyst removal at 38–72 h of incubation. These embryos were selected from a total of 31 which survived for various periods up to the age of 18 days. Embryos older than E8 were anesthetized with Nembutal (approximately 20 mg/kg body weight) prior to sacrifice. Animals were killed by perfusion fixation and/or immersion fixation and were staged according to Hamburger and Hamilton (1951). Their ages will be referred to in days and hours of incubation.

Otocyst Removals

Approximately 400 eggs were incubated for 38–72 h. A lateral window was made in the shell above the embryo and a small drop of sterile 2% neutral red dye in distilled water was applied with a syringe to the embryonic membranes over the right otocyst, using an operating microscope. After staining, the embryos were staged according to the Hamburger and Hamilton (1951) series, and the membranes covering the otocyst were removed with fine forceps. Finely sharpened tungsten needles were used to remove the tissue overlying the otocyst (Fig. 1) and to make cuts on four sides of this structure. The otocyst was then gently peeled from the subjacent tissue and lifted out of the embryo with forceps. Following otocyst ablation, the window in the shell was sealed with surgical tape. The eggs were returned to an incubator and allowed to resume development. Fixed embryos were processed in one of three ways: (1) normal histology; (2) histochemistry for acetylcholinesterase (AchE) activity; and (3) injections with the neuronal tracer DiI.

Normal Histology

Of the operated embryos, 20 were sacrificed at ages between E4.5 and E18 and processed for normal histology. Most of the heads were placed into Carnoy's fluid for 1–2

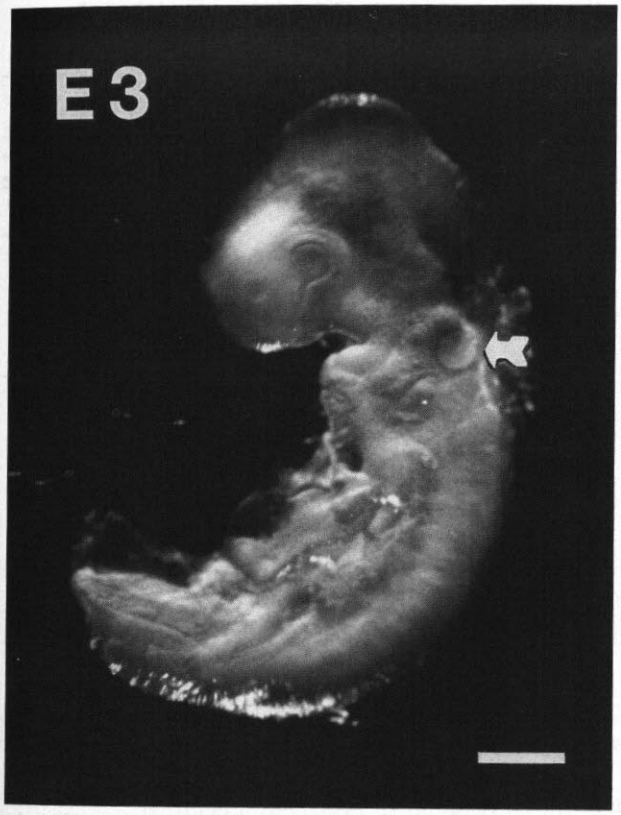

Figure 1 Lateral view of a 3-day-old chick embryo. The arrow indicates the otocyst. Otocyst removals were performed at this age and earlier. Scale bar = 1 mm.

h and postfixed in Bouin's fluid for 3 h. The tissue was embedded in paraffin and sectioned in the transverse plane at 6 μm. Every fourth section was mounted on chrom-alum/gelatine-coated slides and stained with thionin. Two embryos were fixed in 2% paraformaldehyde and 0.5% glutaraldehyde and cryosectioned at 25 μm. In two E10 and one E13 embryo, the number of neurons was determined in the geniculate ganglion on the experimental side and on the unoperated, control side by counting nuclei in every fourth section; these counts were corrected for split cells according to the Abercrombie formula (Konigsmark, 1970). In addition, cell numbers were determined in the geniculate ganglion of one unoperated, age-matched, control embryo. Most cell counts were carried out twice; independently by two investigators. The numbers differed by <5%. The paraffin-embedded, thionin-stained tissue was used for measurements of cell sizes and nuclear sizes of facial and vestibular ganglion cells (Table 1). Measurements were made from about 5% of the cells throughout the ganglion. Cells were sampled randomly by projecting sections in a camera lucida system onto an orthogonal grid with lines separated by 40 μm. The cell sizes and nuclear sizes were measured in all ganglion cells that superimposed with a grid dot, provided that the nucleolus was clearly identifiable in that cell. Because of the small number of facial ganglion cells in the facial canal (63–115), measurements were made twice for these neurons. Sections from one E4.5 embryo were projected in a camera lucida, and facial ganglion cells and the presumptive facial nerve were drawn at 20× magnification.

Acetylcholinesterase Histochemistry

For visualization of cranial ganglia and nerve pathways, nine normal embryos (E2.5–12) and seven operated embryos (E9–11) were processed for AchE activity. A modification (Lynch and Killackey, 1974) of the Koelle method (Koelle and Friedenwald, 1949) was used. Embryos were perfusion fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. After postfixation for 2–4 h, they were cryoprotected in 30% sucrose, and the entire heads were cryosectioned at 30–40 μm in the transverse plane. Sections were collected on two sets of gelatine-coated slides, dried, and one set of sections was stained with thionin. The other set was incubated in acetylthiocholine iodide solution at room temperature according to the protocol of Lynch and Killackey (1974). After 24 h, the incubated sections were developed for 1 min in 1% ammonium sulfide, rinsed, and some of the sections were lightly counterstained with thionin prior to coverslipping. Sections were examined with Nomarski optics on a Leitz Aristoplan microscope.

Tracer Injections

Normal embryos (n = 29; E3–12) and 12 operated embryos (E3–12) were injected with the fluorescent carbocyanine tracers DiI or DiO (Molecular Probes, Eugene, OR). A modification (von Bartheld, Cunningham, and Rubel, 1990) of the "postmortem in vitro" method of Godement, Vanselow, Thanos, and Bonhoeffer (1987) was used. DiI and DiO were dissolved in dimethylformamide (final concentration: 0.5%). After perfusion or immersion fixation in 2% paraformaldehyde and 0.5% glutaraldehyde and overnight postfixation, the tracer was injected into middle-ear regions containing peripheral portions of the facial nerve using a Picospritzer (General Valve Corp.). In embryos with unilateral otocyst removals, injections of DiI or DiO were made on the operated side. In several cases, the injections of DiI were too large and encroached trigeminal territory, missed the facial nerve, or removal of the otocyst turned out to be incomplete. In optimal cases, the injection site included the normal site of the geniculate ganglion and its peripheral branches, the chorda tympani and the paratympanic branch supplying the paratympanic organ (von Bartheld, 1990). Of the 12 experimental embryos, three received optimal injections of tracer into the facial periphery, that is, sufficient but selective, and these cases were used for analysis (Table 2). Embryos injected with tracers were kept in the same fixative solutions for periods of 2–4 months at room temperature or at 37°C. They were cryoprotected overnight in 30% sucrose, embedded in TissueTek OCT medium (Miles), frozen on

Table 1 Cell Sizes and Nuclear Sizes of Ganglion Cells in the Facial and Vestibular Ganglion of Normal Chick Embryos and after Otocyst Removal

	E11 normal[1]	E13 Normal Embryo (88–4311)			E13 Experimental Embryo (88–4307)		
Cranial Ganglion	Cell Size [μm]	*n*	Cell Size [μm]	Nuclear Size [μm]	*n*	Cell Size [μm]	Nuclear Size [μm]
Geniculate	8.1	20	16.2	9.4	29[2]	15.7	8.1
Deviated facial	—	—	—	—	20	15.0	7.8
Vestibular	6.1–6.8	25	12.9	8.0	56[2]	12.4	6.5
Facial canal	8.0	10	18.2	9.6	9[2]	17.0	9.3

n = number of ganglion cells measured.
[1] Data from D'Amico-Martel, 1982.
[2] Measurements from side contralateral to otocyst removal.

dry ice and cryosectioned at 30–40 μm in the transverse plane. Sections were collected on gelatine-coated slides and observed without a coverslip on a fluorescence microscope equipped with standard rhodamine and fluorescein isothiocyanate (FITC) filters. Labeled structures were documented on TMAX film using exposure times

Table 2 Deviation of the Facial Nerve after Otocyst Removal in Chick Embryos

Animal No.	Age at Surgery (h)	Age at Sacrifice (days)	Extent of Removal[2] V:	C:	Analysis	Facial Pathway
C 19-1[1]	40–45	E8	100%		NH	G
C 18-2[1]	45–49	E10	100%		NH	—
C 34[1]	45–49	E8	100%		NH	—
C 29-6[1]	45–49	E8	100%		NH	—
89-2335	38–40	E10	V: 100%	C: 98%	AchE	—
89-2337	40–45	E11	100%		AchE	—
89-2346	42–46	E9	V: 90%	C: 100%	AchE	—
89-2347	48–50	E9	V: 50%	C: 100%	AchE	—
89-2348	48–50	E9	V: 40%	C: 100%	AchE	—
89-2336	48–50	E10	100%		AchE	T
89-2328	50–53	E4.5	100%		NH	—
89-2332	50–53	E10	100%		AchE	T
89-2329	50–53	E4.5	100%		NH	T
89-2302	70	E9.5	V: 50%	C: 100%	DiI	—
89-2303	70	E9.5	V: 100%	C: 60%	DiI	T
89-2305	70	E11.5	100%		DiI	T
88-4306	72	E13	100%		NH	—
88-4307	72	E13	100%		NH	T
88-4313	72	E14	100%		NH	—
88-4314	72	E8	100%		NH	—
88-4316	72	E16	100%		NH	—
89-4846	72	E17	100%		NH	T
89-4847	72	E17	100%		NH	T
88-4315	72	E18	100%		NH	—

AchE = acetylcholinesterase histochemistry; C = cochlear part of inner ear; DiI = DiI injections; G = deviation via glossopharyngeal ganglion; NH = normal histology; T = deviation via trigeminal ganglion; V = vestibular part of inner ear; — = pathway at normal level.
[1] Data from Yntema (1944). The ages of Yntema's embryos at the time of surgery are inferred from somite counts (Hamburger and Hamilton, 1951).
[2] Estimates when <100%.

Figure 2 Transverse section through the brain stem of a chick embryo after 11.5 days of incubation. The otocyst was removed at E3 on the left side, and the facial nerve was injected with the fluorescent tracer DiI. The eighth nerve (N VIII) is absent on the left side (compare with right side), but the facial nerve (N VII) enters the brain stem at its normal site despite its peripheral deviation to the level of the trigeminal ganglion [Fig. 3(B)]. Many neurons are labeled retrogradely in the facial motor nucleus (M VII, arrowheads) at this level and farther caudally [Fig. 5(D)]. Scale bar = 0.5 mm.

of 45 s to 3 min. Facial nerve pathways from three successful cases were projected, drawn, and analyzed in serial charts.

RESULTS

We will first describe some features of normal development of the facial nerve that will be relevant for the interpretation of the experimental data.

Normal Development

Following injections of DiI into the peripheral nerve at E4–5, many geniculate facial ganglion cells were retrogradely labeled. At this age, no label was detected in motor neurons. It is unlikely that the failure to label motor neurons was due to the longer pathway, for at later stages, and with the same diffusion time, facial motor neurons were heavily labeled (Fig. 2). Facial motoneurons were first labeled at E6 following injections of DiI into peripheral targets of the facial nerve. Thus, processes from the geniculate ganglion cells appear to arrive in peripheral targets earlier than the motor nerve fibers.

At E7, cartilage begins to form which separates the vestibular and proximal facial ganglion from the geniculate facial ganglion in the middle ear. The facial nerve fibers enter the brain in the rostral portion of the seventh/eighth nerve complex. The geniculate ganglion, the proximal ganglion, and the facial canal are found at the same level of the cranium in the transverse plane [Fig. 3(A)]. Within the brain, the facial nerve separates from the vestibulocochlear nerve. The targets of sensory facial nerve fibers could not be determined with certainty in the DiI preparations because it was difficult to distinguish fibers of passage from terminal fibers in the cryosections; it is known from previous studies that the facial nerve fibers project to the nuclei of the solitary tract (Ganchrow, Ganchrow, and Gentle, 1986). Eighth nerve fibers join the facial nerve in the facial canal and enter the brain stem with the facial nerve, but proceed to vestibular and auditory brain stem nuclei.

For comparisons with experimental data, it was necessary to investigate the normal numbers and the morphology of ganglion cells in the developing geniculate ganglion. This ganglion comprises up to four distinct subdivisions: the geniculate ganglion proper, the cells scattered in the facial canal

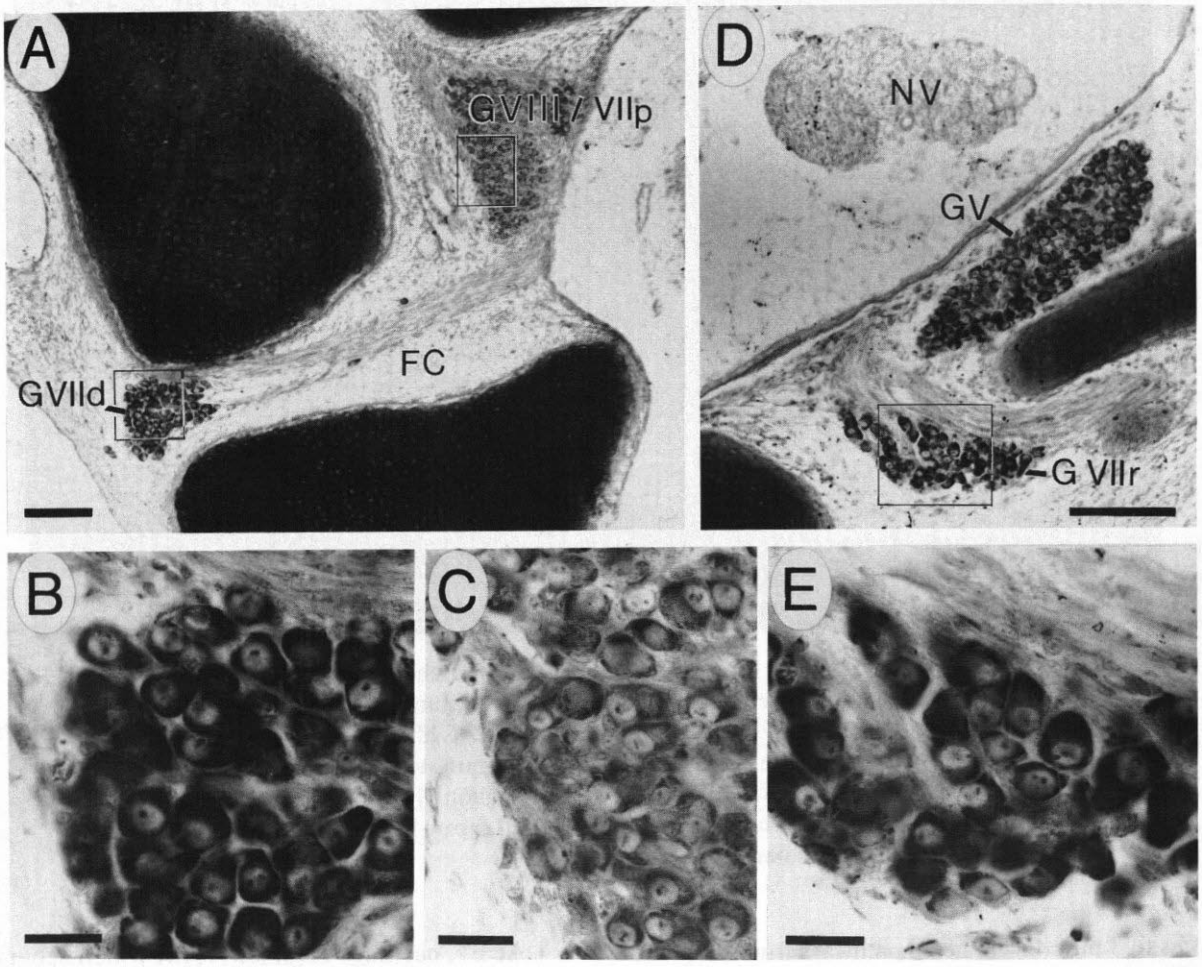

Figure 3 Transverse sections through the normal facial nerve (A–C, left side) and abnormal facial nerve and ganglion (D, E, right side) of a 13-day-old chick embryo following otocyst removal at E3. Paraffin sections were stained with thionin. Medial is towards the center. (A) Micrograph shows the location of the normal geniculate (distal) facial ganglion (G VIId), the facial canal (FC), and the eighth nerve ganglion (G VIII). The G VIII also contains the proximal facial ganglion cells (G VIIp). Neurons of the G VIId and the G VIII/VIIp are shown at higher magnification in (B) and (C), respectively. (B) Note dark Nissl staining of the large G VIId cell bodies. (C) Note light Nissl staining of the small G VIII/VIIp cell bodies. (D) Micrograph shows the location of the deviated, "rostral" facial ganglion (G VIIr) at the level of the trigeminal ganglion (G V) and the trigeminal nerve (N V). This section from the opposite side of the head shown in (A) was mounted and stained on the same slide. The boxed area is shown at higher magnification. (E) Note dark Nissl staining of the large G VIIr cell bodies which is similar to the staining in G VIId (B), but differs from that in the G VIIp (C). Scale bars = 100 μm (A, D); 20 μm (B, C, E).

(D'Amico-Martel, 1982), the paratympanic ganglion (Benjamins, 1925; Oldenstam, 1925; von Bartheld, 1990), and, often, a small number of ganglion cells along the facial nerve in the middle ear associated with neither of the above divisions. In one E13 embryo, we counted a total of 609 ganglion cells in the distal (geniculate) facial ganglion proper, 115 ganglion cells scattered in the facial canal, and 81 ganglion cells in the paratympanic ganglion (Table 3).

Neural crest-derived and placode-derived (geniculate) ganglion cells show a transient cytological dichotomy on the basis of size as well as the intensity of Nissl stain in chick embryos (D'Amico-Martel and Noden, 1983), with placode-derived cells being larger and darkly stained, whereas neural crest-derived cells are smaller and lightly stained. At E13, all ganglion cells of the geniculate ganglion proper stained darkly, and averaged 16.2 μm in diameter [Fig. 3(B)]. Their nu-

Table 3 Number of Ganglion Cells in the Distal Facial Ganglion on the Normal Side and the Side with Otocyst Removal in 10- and 13-Day Old Chick Embryos

Case Number	Age at Otocyst Removal [h]	Age at Sacrifice [days]	Total Number	(%)	Geniculate Ganglion	Facial Canal	Paratympanic Ganglion	Central Pathway
89-2336	48–50	10	norm. 749		575	76	86	2
			exp. 709	(95%)	648	—	55	6
89-2332	50–53	10	norm. 894		744	63	84	3
			exp. 858	(96%)	747	—	87	24
89-4307	72	13	norm. 770		556	65	144	5
			exp. 535	(69%)	437	—	53	45
88-4311	–	13	norm. 805	—	609	115	81	0

exp. = experimental side; norm. = normal side.

cleus measured 9.4 μm. The vestibular and the proximal facial ganglion cells cannot be distinguished morphologically (D'Amico-Martel, 1982). They averaged 12.9 μm in diameter and 8.0 μm in nuclear diameter and showed pale Nissl stain [Fig. 3(C)]. The ganglion cells in the facial canal stained darkly and were larger than either the vestibular or the geniculate ganglion cells (diameters averaged 18.2 μm, Table 1). Otherwise, the ganglion cells in the facial canal resembled the ganglion cells of the geniculate ganglion proper [Fig. 3(A)].

Otocyst Removals

Surgery of the inner-ear anlage resulted in two types of embryos, those with complete removal of the inner-ear anlage, and those with partial removal. As is evident in Table 2, partial removal of the otocyst was more frequent in embryos that had undergone surgery at early stages (38–70 h) than those that were operated after 70 h of incubation. These differences may be due to technical aspects of the surgery.

Following complete removal of the otocyst, the inner-ear labyrinth was absent. In these cases and in some cases with partial otocyst removals, the facial nerve followed one of two distinct pathways. In seven out of 14 cases with complete removal of the otocyst, it entered the cranium through a relatively normal facial canal. In these cases, the geniculate ganglion cells formed a more-or-less compact ganglion at the usual level, but no distinct proximal facial ganglion could be identified. The facial ganglion cells and nerve fibers showed an abnormal rostral deviation towards the trigeminal ganglion in eight experimental embryos (seven embryos with complete otocyst removals and one embryo with complete removal of the vestibular part but persistence of parts of the cochlea) [Figs. 3(D,E), 4(A,B), 5, 6; Table 2]. All of these embryos were sacrificed at ages between E4.5 and 17. In one case, the facial ganglion virtually fused with the trigeminal ganglion. In two cases, the deviated ganglion formed two more-or-less continuous portions, one further proximal, the other further distal [Fig. 4(B)], but both were located at the trigeminal level. In five cases, the deviated cell bodies formed a single, compact ganglion in the trigeminal foramen [Fig. 3(D)] or within the cranium adjacent to the trigeminal ganglion [Fig. 4(A)]; only few cell bodies were distributed along the peripheral and central trajectory of the facial nerve. The central fibers of the facial nerve appeared to enter the brain stem at the normal facial level, however, it was not possible, in the tissue processed for normal histology or for acetylcholinesterase activity, to determine if all facial nerve fibers projected to the facial level, or if some or many facial nerve fibers entered the brain stem with the trigeminal nerve fibers. This question was addressed by injections of tracers (see below).

Deviated ganglion cells showed intense Nissl staining [Fig. 3(E)]. The intensity of staining resembled the staining of cells in the placode-derived geniculate ganglion [Fig. 3(B)] and differed from the pale staining of cells in the vestibulocochlear ganglion and neural crest-derived proximal facial (root) ganglion cells at this age [E13, Fig. 3(C)]. The diameter of the deviated facial ganglion cells averaged 15.0 μm at E13; their nuclei averaged 7.8 μm in diameter. These parameters were very similar to those of the geniculate ganglion from the contralateral, normal side (15.7 μm cell size; 8.1 μm nuclear size), but differed significantly from the size of contralateral (normal) vestibular/proximal

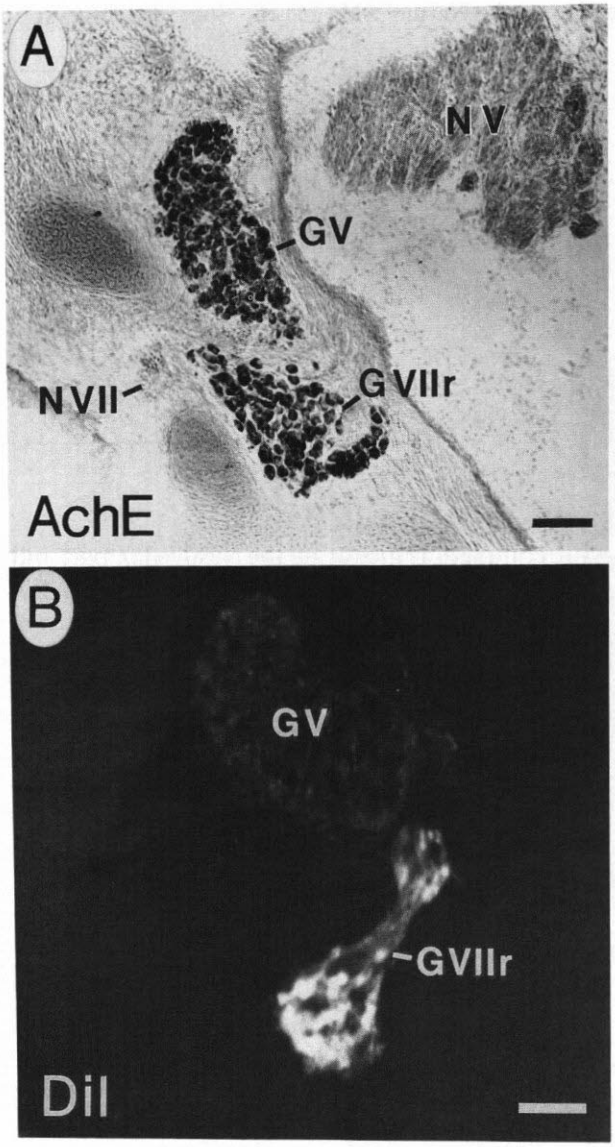

Figure 4 Transverse sections through the abnormal facial ganglion (G VIIr), which is deviated rostrally adjacent to the trigeminal ganglion (G V) following otocyst removal in the 3-day-old chick embryo. (A) Section was processed for acetylcholinesterase activity. Age: E10. N VII, facial nerve, other abbreviations as per Figure 3. (B) Section shows the labeled G VIIr following injections of the fluorescent tracer DiI into the peripheral facial nerve. Age: E9.5.

facial ganglion cells (cell size: 12.4 μm; nuclear size: 6.5 μm, Table 1). Facial and acoustic ganglion cells on the unoperated side of experimental embryos differed little in cell size from unoperated, age-matched control embryos (Table 1). The deviated facial ganglion cells thus were most likely of placodal origin rather than neural crest origin (cf. Hamburger, 1961; D'Amico-Martel and Noden, 1983).

To determine how disruption of the normal pathway may affect cell numbers, we counted the ganglion cells in the geniculate ganglion of the normal side and in the deviated facial ganglion of the experimental side in three embryos at E10 and E13. The total number of ganglion cells in the geniculate ganglion was 535–858 on the experimental side, and 749–894 on the normal side (Table 3). Otocyst removal thus appeared to cause a loss of geniculate ganglion cells between 4% and 31%. Apparently, the large majority of geniculate facial ganglion cells survived the otocyst removal. The similarity in numbers of cells in the distal facial ganglion from a normal embryo (805) and the control side of operated embryos (749, 770, and 894) indicates that windowing and unilateral otocyst removal does not affect cell numbers in the *contralateral* geniculate ganglion of experimental embryos (cf. Fisher and Schoenwolf, 1983). There may be a small effect of the surgery on the size of the ganglion cells and their nuclei, but these differences may also be due to minor differences in the age of the embryos.

Translocation of the geniculate facial ganglion to the trigeminal level occurred in eight of 17 embryos operated between 48 and 72 h of incubation (Table 2). This type of translocation was not seen in embryos operated earlier, between 38 and 48 h, neither in our material (n = 3) nor in that of Yntema (1944) (n = 4), as listed in Table 2. Timing of the surgery may be a relevant factor in the induction of the deviation of the facial nerve (see Discussion).

In six animals, the surgery resulted in partial otocyst removals (Table 2). Two of these cases showed complete or nearly complete removal of the neurogenic portions of the otocyst; in four other embryos, only vestibular (n = 2) or only cochlear portions (n = 2) of the inner ear remained. Four of the experimental embryos with partial otocyst removal were processed for acetylcholinesterase activity which labels embryonic ganglion cell bodies and nerve fibers (Strumia and Baima-Bollone, 1964). This procedure facilitated the evaluation of the success of the surgery, because single acoustic ganglion cells were easy to identify in rudimentary inner ears. In one case with persistence of parts of the inner ear including the vestibular nerve, no facial nerve deviation was seen (Table 2). In another case in which the vestibular nerve was absent but the cochlear nerve persisted (no. 89-2303, see below), the facial nerve deviated towards the trigeminal ganglion.

To determine whether or not some deviated fa-

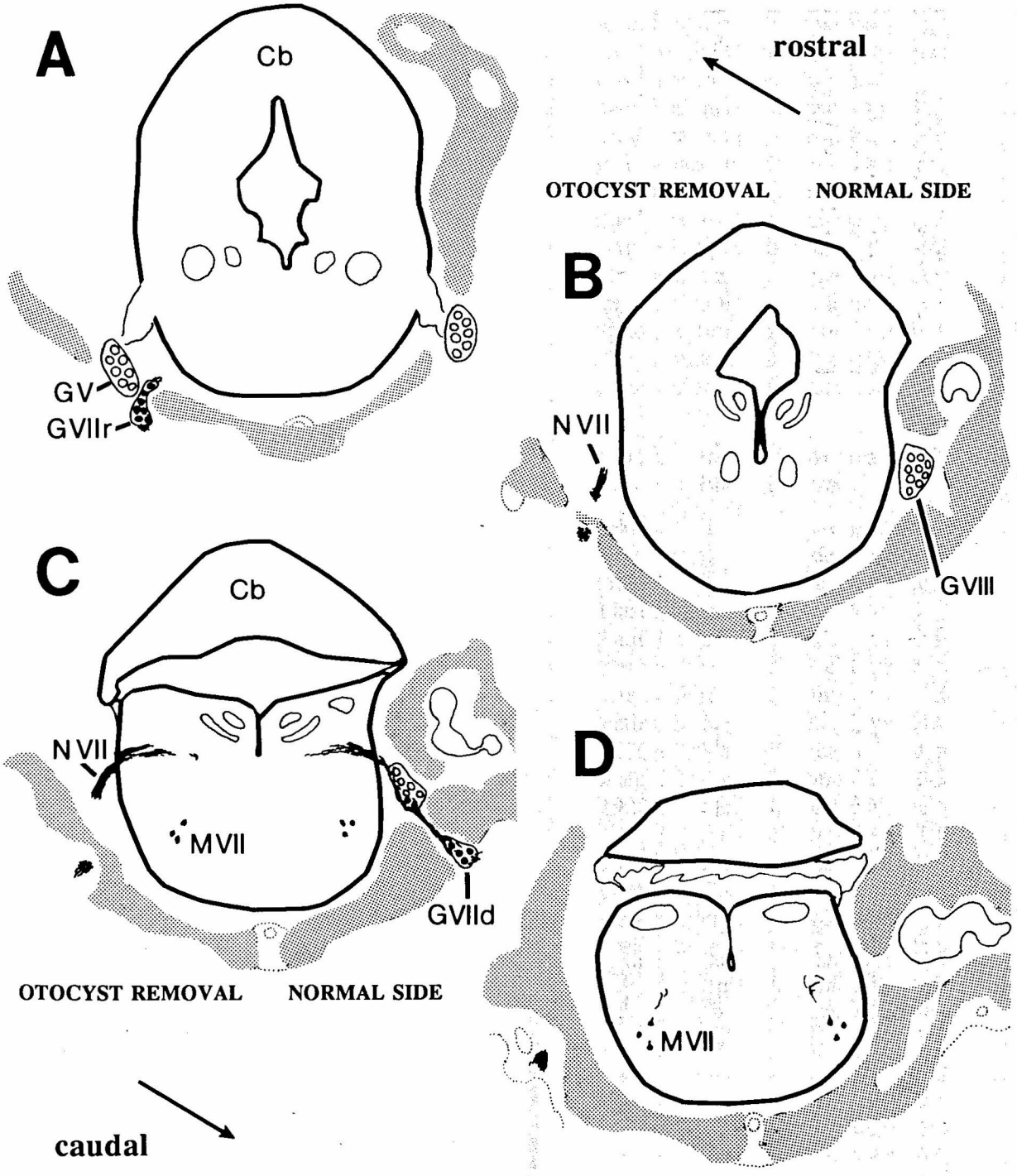

Figure 5 Rostral-to-caudal (A–D) charts of the abnormal (left) and normal (right) facial nerve pathway in a chick embryo of 11.5 days of incubation. Facial nerve pathways were visualized following injections of DiI into the peripheral facial nerve. Labeled neurons and pathways are drawn in black; unlabeled ganglion cells are depicted as open circles. Following otocyst removal, the facial nerve deviates rostrally and enters the cranium through the trigeminal foramen. Cb = cerebellum; other abbreviations as per Figures 2–4.

cial nerve fibers intermingle with trigeminal fibers and enter the brain with the trigeminal nerve, DiI was injected into the peripheral facial nerve in fixed tissue of operated embryos. In three embryos the concentration of DiI was abundant throughout the injection site, but apparently did not encroach into tissue innervated by the trigeminal nerve, since no trigeminal ganglion cells were labeled.

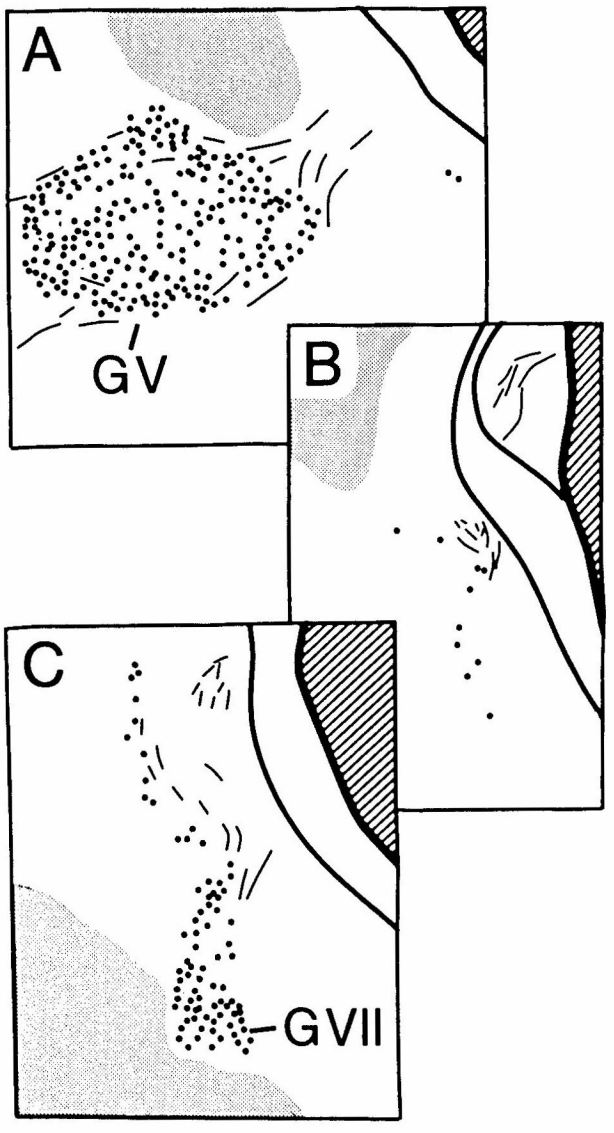

Figure 6 Camera lucida drawings of trigeminal and facial ganglion cells and nerve fibers in a 4.5-day-old chick embryo following removal of the otocyst at 50–53 h. Three transverse sections from the experimental side at levels from rostral to caudal (A–C) are shown; the distance between A and B and B and C is 100 and 50 μm, respectively. Black dots indicate ganglion cells; thin lines represent fascicles of nerve fibers, thicker lines outline the cranium and brain (hatched). Note that cells of the facial ganglion (G VII) and presumptive facial nerve fibers extend rostrally towards the trigeminal ganglion. Abbreviations as per Figure 3.

These cases were used for detailed analysis (Table 2; Fig. 5). Two cases revealed the course of the deviated facial nerve fibers in the cranium and their pathway towards central targets in the brain. The facial nerve fibers turned rostrally and penetrated the cranium with the trigeminal nerve. The facial ganglion cell bodies lay immediately adjacent to the trigeminal ganglion [Fig. 4(A,B)]. Virtually all cells in the deviated ganglion were labeled with DiI [Fig. 4(B)]. The facial nerve fibers did not enter the brain at the level of the trigeminal nerve, but turned caudally, ran adjacent to the brain to the normal site of entrance of the facial nerve into the medulla [Figs. 2; 5(C)], entered there, and proceeded towards their normal targets in the brain. The resolution of the DiI label was not sufficient to identify terminals in putative target structures of the facial nerve (Ganchrow et al., 1986). In one case, two individual facial nerve fibers could be followed for a short distance (40–50 μm) along the trigeminal nerve towards and into the CNS (data not shown). The course of these two nerve fibers obviously was aberrant for they did not proceed within the CNS. It is unlikely that absence of label in these two fibers was due to fading of the dye, because in the same preparation, numerous facial nerve fibers were labeled continuously and could be followed to their entrance into the brain at the facial level and beyond (Figs. 2, 5).

Many facial motor neurons were labeled in the brain stem following injections of DiI [Figs. 2, 5(C,D)]. Motor fibers followed the rostral loop of the deviated sensory facial nerve fibers. No separate facial nerve pathway was found to project towards the periphery. It cannot be concluded that all facial motor neurons project to their normal targets, because we do not know whether all motor neurons were retrogradely labeled with DiI. The motor neurons that were labeled, however, did not have processes that intermingled with trigeminal nerve fibers and they did not follow trigeminal pathways.

Injections of DiI included two fortunate cases with *partial* otocyst removals. In animal no. 89-2303, the vestibular part of the inner ear was removed completely at 70 h, but large parts of the cochlear part remained. In this case, the facial nerve deviated to the trigeminal ganglion. In animal no. 89-2302, the cochlear part of the inner ear was removed completely at 70 h, but large parts of the vestibular division remained: in this case, the facial nerve did not deviate. Apparently, surgery that eliminated the vestibular part of the otocyst caused a deviation of the facial nerve, but surgery that was restricted to the cochlear part of the otocyst did not interrupt the normal development of the facial nerve.

To determine if translocation of facial ganglion cells is a primary or secondary event, we completely removed the otocyst at 50–53 h and examined the facial nerve at 4.5 days of incubation. At

E4.5, sensory facial axons have entered the hindbrain in normal chick embryos (Vogel and Davies, 1991) and after otocyst removal (Petralia, Gill, and Peusner, 1991; R. S. Petralia, personal communication). In one of the E4.5 embryos, the facial ganglion and nerve was located at the same level on the two sides. The other embryo showed an assymmetry: the facial ganglion was located closer to the trigeminal ganglion (distance of 150 μm) on the experimental side than on the normal side (400 μm), and many facial ganglion cells extended dorsally and rostrally towards the trigeminal ganglion on the experimental side (Fig. 6). The facial nerve also appeared to project farther rostrally; however, the identity of the fibers indicated in Figure 6 was not verified. As will be discussed below, these data are consistent with the hypothesis that ganglion cell bodies trail their deviated axons to an abnormal rostral position.

DISCUSSION

The ability of developing axons to grow along particular pathways and to innervate specific targets is an essential feature of neural circuit formation. Various factors may provide guidance cues for navigating axons (Jacobson, 1978; Purves and Lichtman, 1985; Palka, 1986). To infer possible guidance mechanisms, one can manipulate the normal pathway and determine pathfinding abilities under altered circumstances, for example, after transplanting projection neurons to an ectopic site or into a host of a different age (Ghysen, 1978; Constantine-Paton, 1983; Murphey, Johnson, and Sakaguchi, 1983; Harris, 1984, 1986; Honig et al., 1986), by inserting a barrier in the normal pathway (Moody and Heaton, 1983c), and by eliminating pioneer fibers or intermediate targets (Edwards, Chen, and Berns, 1981; Bentley and Caudy, 1983; Kuwada, 1986; Chitnis and Kuwada, 1991; Bernhardt, Nguyen, and Kuwada, 1992; Pike et al., 1992). In vertebrates, studies of navigating nerve fibers have focused on pathfinding of motor axons (Lance-Jones and Landmesser, 1980, 1981; Landmesser, 1980; Purves and Lichtman, 1985) and peripheral pathfinding of neural crest-derived sensory ganglia (Lewis, Chevallier, Kieny, and Wolpert, 1981; Landmesser and Honig, 1986; Honig et al., 1986; Scott, 1986), but little is known about central pathfinding abilities of sensory cells of placodal origin (Székely, 1959; Hamburger, 1961; Constantine-Paton, 1983).

Our study shows that placode-derived geniculate facial ganglion cells project towards their normal targets in the CNS when their neurites are induced to enter the cranium (or neural tube) with the trigeminal nerve. Disruption of the earliest fibers (and presumably retrograde degeneration of their cell bodies) in the geniculate ganglion does not abolish pathfinding abilities of regrowing or subsequent axons that proceed towards the cranium. Apparently, pathfinding abilities are not restricted to the normal pioneers of the geniculate ganglion. The pathway that facial nerve fibers follow after disruption of their pioneers indicates that these fibers make pathway choices and may respond to a variety of guidance cues.

Normal Development of the Facial and Vestibulocochlear Nerves

Facial Nerve. The geniculate ganglion is derived from the epibranchial placode. Placodal cells proliferate after the second day of incubation and peak neuroblast migration occurs at 60–64 h (D'Amico-Martel and Noden, 1983). The ganglion cells grow processes peripherally and centrally; the first central fibers penetrate the brain around E3 (Windle and Austin, 1936; Petralia and Peusner, 1991; Vogel and Davies, 1991). The early facial nerve pathway is separate from the otocyst (E2–3) (Holmdahl, 1928), but at E3, the facial nerve pathway joins and intermingles with the eighth nerve and the vestibular ganglion (Goronowitsch, 1893; Romanoff, 1960; Knowlton, 1967). Facial nerve fibers sometimes run both through, and along the ventral aspect of the vestibular ganglion (D'Amico-Martel, 1982). Communicating branches form at E3 rostrally with the trigeminal ganglion, and caudally with the glossopharyngeal ganglion (Kuratani, Tanaka, Ishikawa, and Zukeran, 1988), and a transient posterior branch projects subjacent to the otocyst (Kuratani et al., 1988). The neural crest-derived, proximal (root portion) of the facial ganglion does not proliferate until E4–5 (D'Amico-Martel, 1982). Motor fibers of the facial motor nucleus leave the neural tube at 79 h (Bok, 1915) and enter the ganglion primordium (Tello, 1923); however, our data indicate that they do not proceed into the periphery prior to E6.

Vestibulocochlear Nerve. Ganglion cells of the eighth nerve proliferate and migrate from the ventromedial part of the otocyst (Alvarez, Martin-Partido, Rodriguez-Gallardo, Gonzales-Ramos, and Navascués, 1989). Birthdates of these cells range from E2 to E5 in the vestibular ganglion, and from

E4 to E7 in the cochlear ganglion (D'Amico-Martel, 1982). In DiI-labeled material, the earliest eighth nerve fibers penetrate the CNS at E2–3 (cf. Windle and Austin, 1936; Petralia and Peusner, 1991; Vogel and Davies, 1991; von Bartheld et al., 1991).

These data suggest that the first vestibular and the first facial nerve fibers from the distal facial ganglion arrive at about the same time in the CNS. Neither of these two components appears to pioneer a pathway that is only followed by the other nerve. Because normal facial pathways can form in cases with complete otocyst removal, the vestibular nerve obviously is not essential in the formation of the facial pathway. The occurrence of transient communicating branches with the trigeminal and glossopharyngeal ganglia and the variability of courses of facial nerve fibers through the vestibular ganglion (or vestibular anlage) may have important consequences for the development of the facial pathway following otocyst removals.

Hypothetical Mechanism of Deviation

The facial nerve projects into the brain stem in the absence of the otocyst, the inner-ear anlage (Yntema, 1944; Petralia et al., 1991). We show that the facial nerve deviates in some, but not all cases with otocyst removal, and that complete otocyst removal does not always result in a deviation of the facial nerve (Table 2). How can two distinct types of facial pathways be explained in the experimental cases? One simple explanation is that the facial nerve fibers are disrupted only in embryos in which the facial nerve fibers penetrated the early vestibular ganglion (part of the otocyst anlage), but not in embryos in which the facial nerve fibers ran *outside* along the vestibular ganglion. Such variability in facial nerve trajectories has been described in normal chick embryos (D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983). This explanation is consistent with the observation that a deviated facial nerve occurs more often after surgery in older embryos than after surgery in younger embryos. Deviation via the trigeminal ganglion was not observed in chick embryos operated prior to 48 h of incubation (Table 2). At this time the facial pathway has not yet merged with the otocyst in the chick embryo (Goronowitsch, 1893; Holmdahl, 1928; Romanoff, 1960; Knowlton, 1967).

Another explanation, not mutually exclusive with the first one, is that transient communicating branches with the trigeminal ganglion may be important (Kuratani et al., 1988). Remaining or forming communicating branches may guide growing facial nerve fibers when their normal central pathway is disrupted. The transient branch communicating with the glossopharyngeal ganglion may be more likely to be affected by otocyst removal than the one connecting with the trigeminal ganglion. This may be the reason why translocation usually occurs towards the trigeminal rather than the glossopharyngeal ganglion. The trigeminal nerve enters the brain earlier (48–52 h) (Heaton and Moody, 1980) than the vestibular/facial nerve (52–64 h) and thus may guide facial nerve fibers.

Recent studies showed that disruption of the homeobox gene *hox* 1.6 reduces the size and development of the otocyst and results in a rostral displacement and fusion of the facial ganglion and nerve with the trigeminal in homozygous mice (Chisaka, Musci, and Capecchi, 1992). It is intriguing that surgical manipulations (otocyst removal, our study) and genetic manipulations can lead to very similar phenotypes, and one is tempted to speculate that the two manipulations may have a common cause, for example, removal of a *hox* gene product that is crucially involved in the initial development of facial nerve fibers.

Deviated facial nerve fibers appear to make at least three different choices in the selection of their pathway (Fig. 7): first, to grow towards the trigeminal ganglion; second, not to continue their course with the trigeminal nerve centrally, but to turn back caudally within the cranium; and third, to enter the medulla at the usual entrance of the facial nerve. This pathfinding behavior is consistent with the notion that multiple local cues may guide navigating axons. So-called guide-post neurons have been assigned a role as "stepping stones" during pathway formation in some invertebrate systems (Ho and Goodman, 1982; Bentley and Caudy, 1983; Caudy and Bentley, 1986). Such guidance cues, however, may also be inherent in the matrix itself rather than being expressed by guide-post cells proper (Blair and Palka, 1985; Schubiger and Palka, 1985; Blair, Murray, and Palka, 1985).

Motor Nerve Fibers

In cases with deviated facial nerves, both sensory and motor facial nerve fibers make the same detour; there is no evidence for a distinct facial motor nerve pathway. May outgrowing motor axons rather than ingrowing sensory nerve fibers pioneer the deviating facial pathway? This is unlikely for the following reasons. The facial sensory fibers nor-

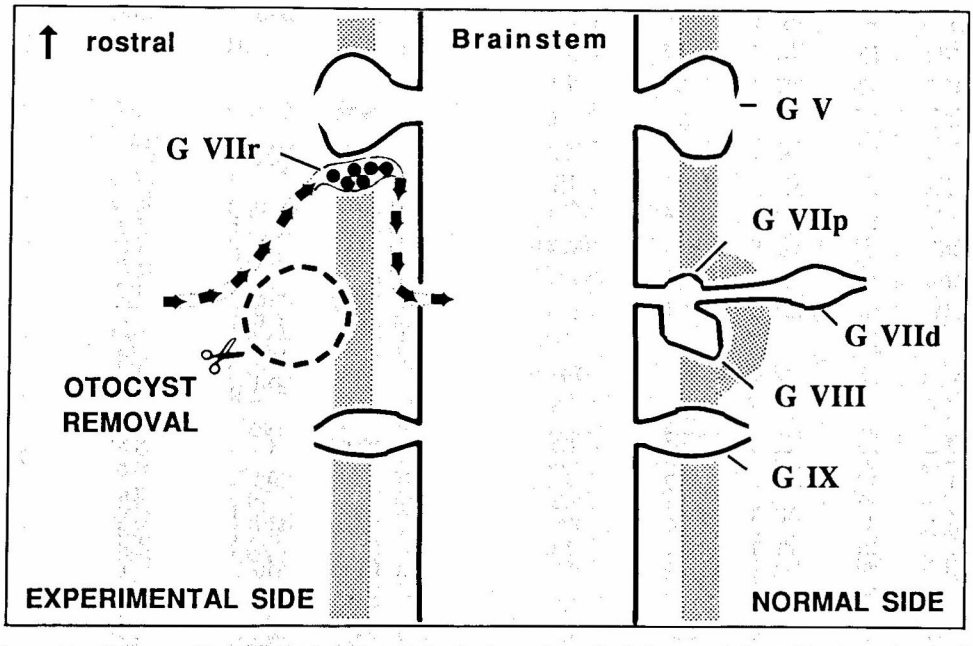

Figure 7 Cartoon illustrates the rostral deviation of the facial nerve (short black arrows) after otocyst removal in chick embryos. Schematic drawing in a horizontal plane. Rostral is to the top, caudal is to the bottom. G VIIp, proximal part of facial ganglion; G IX, glossopharyngeal ganglion; other abbreviations as per Figure 3.

mally enter the medulla *before* or at the same time as the motor fibers exit (Bok, 1915; Windle and Austin, 1936; Petralia and Peusner, 1991; present study). Trigeminal motor axons halt axon outgrowth when the ingrowth of sensory fibers is prevented or delayed (Moody and Heaton, 1983c), and trigeminal motor axons innervate their appropriate muscles only when they make prior contact with the sensory trigeminal ganglion (Moody and Heaton, 1983a).

Pathfinding properties appear to differ between cranial (trigeminal/facial) nerves and trunk (spinal) nerves; in the latter, the motor axons pioneer the path, and the neural crest-derived sensory neurons follow the motor fibers (Landmesser and Honig, 1986; Honig et al., 1986), whereas in the trigeminal nerve, sensory axon ingrowth (to the CNS) and outgrowth (to the periphery) precedes the motor outgrowth at least initially (Noden, 1980; Moody and Heaton, 1983c; Moody, Quigg, and Frankfurter, 1989). Therefore, it is more likely that the motor axons influence sensory fibers *after* they have deviated on their way towards the medulla.

Neural Crest

The neural crest has been implicated in the formation of a normal trigeminal ganglion (Yntema, 1944; Hamburger, 1961; Noden, 1978; Moody and Heaton, 1983b). Hamburger (1961) reported that placodal trigeminal ganglion cells from which neural crest had been removed are competent to form normal peripheral pathways, but they do not send processes into the CNS when the neural crest is absent. In the deviated facial ganglion of chick embryos, ganglion cells appear to be of the geniculate (placode-derived) type [Fig. 3(B,E)], and there is no evidence for a contribution of neural crest-derived elements. We never observed rudimentary proximal (neural crest-derived) ganglia after complete otocyst removals, as Hamburger (1961) did in similar experiments with the trigeminal nerve. This indicates that the neural crest which normally forms the proximal facial ganglion either was completely removed or that it failed to develop after otocyst ablation. If the neural crest is essential in specifying central projections of placodal cells, then one would expect that placodal ganglia fail to establish their normal central projections after disruption of the neural crest. This, however, was not the case.

In our study, the trigeminal ganglion appears to guide aberrant facial nerve fibers, but does not induce them to proceed with the trigeminal nerve and to enter the CNS at this "wrong" level. Previously, it has been described that abnormal sympathetic fibers, when induced to grow into the trigemi-

nal ganglion, likewise failed to enter the brain stem via the trigeminal nerve, although the same type of aberrant (sympathetic) fibers entered the medulla via more caudal (trunk) ganglia and nerves (Menesini-Chen, Chen, and Levi-Montalcini, 1978). In this respect, the trigeminal nerve appears to differ from trunk (spinal) nerves.

Lack of Sprouting to Deprived Targets

Our results indicate that innervation of the normal targets of the facial nerve does not require a normal peripheral pathway of this nerve. Target specificity thus does not depend on a stereotypic innervation pattern. However, may facial nerve fibers expand their central target territories following otocyst removal and deprivation of afferent input to auditory and vestibular nuclei? It has been proposed in an evolutionary context that targets retain an "affinity" to their ancestral source of innervation, and that this affinity can be reactivated when the target is deprived of its normal afferents (Ebbesson, 1984). The lateral line and the inner-ear sensory organs have been hypothesized to derive from a common ancestral sensory system (for a critical review of the acoustico-lateralis hypothesis, see Northcutt, 1980). The facial nerve contains a "lateral line" component, the paratympanic nerve that supplies a hair cell organ in the avian middle ear (von Bartheld, 1990). Otocyst removal does not disrupt the development of the paratympanic organ (Yntema, 1944), and our injections of DiI into the facial periphery labeled the paratympanic organ and nerve in the middle ear. The facial (paratympanic) nerve did not sprout beyond its normal target area to innervate auditory and additional vestibular brain stem nuclei when these nuclei were deprived of their normal input. These findings confirm similar conclusions on the absence of lateral line nerve sprouting into "deprived" acoustic brain stem nuclei after otocyst removal in amphibians (Fritzsch, 1990). Deprivation of normal afferent input does not appear to alter target specificity in the seventh/eighth nerve. The forces that guide growing axons to their appropriate targets can resist major surgical manipulations.

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